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Comparison of two inoculation methods for *Microsporum canis* culture using the toothbrush sampling technique

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Background

The toothbrush method is an effective method for obtaining material for fungal cultures. However, the correct technique for inoculation onto the agar surface does not appear to have been formally studied

Hypothesis/objectives

This study compared two inoculation techniques; the first involved pressing the toothbrush onto the plate surface (procedure A), and the second involved pressing the toothbrush onto the agar, as well as transferring hairs and scales entrapped in the bristles (procedure B).

Animals

A total of 26 cattery-housed cats were sampled using the toothbrush technique. An individually-packaged new toothbrush was longitudinally combed for 3 min over the hair coat of each cat.

Methods

The toothbrushes from each cat were then randomized to procedure A or B and the investigator was blinded to inoculation technique. Cultures were performed on a medium specific for dermatophytes. Results were compared considering the number of positive plates along with other parameters such as the presence and abundance of colonies of dermatophytes and contaminant moulds.

Results

A total of 21 cats were culture-positive for *Microsporum canis*. Procedure A allowed a significantly higher number of positive plates (20/21) to be obtained compared with procedure B (7/21). These results were mainly due to the higher plate invasion by contaminant moulds, which was evident using procedure B.

Conclusions and clinical importance

This study provides evidence that fungal cultures should be performed by pressing toothbrushes onto agar plates without including hair or scales.

Introduction

Dermatophytosis is a common fungal infection of cats, with *Microsporum canis* considered to be the most important etiological agent.[1] This fungus is found worldwide and plays an important zoonotic role. In some countries, *M. canis* tends to surpass anthropophilic dermatophytes as a cause of human infections.[1] Dermatophytosis can present with a wide variety of clinical signs; therefore, confirmation of infection relies on results from different diagnostic tests. Fungal culture is normally considered the test of choice[2], and sampling techniques for culture vary according to the situation.[3,4] The "toothbrush method" is recommended in cats with generalized lesions or subclinical infections.[4] This represents a variant of the method originally described by McKenzie *et al*.[5], who employed hairbrushes to detect scalp dermatophytosis in children. This method involves combing a human toothbrush (considered mycologically sterile while in its packaging[4]) over the entire hair coat in order to accumulate hair and keratin debris, followed by pressing onto the surface of the culture plate.[4] While this method is widely quoted[1,2,6,7] and used, the correct inoculation technique onto the agar surface has not been formally studied.[4] Specifically, since collected hairs tend to remain entrapped in the bristles despite repeated stabbing onto the medium surface, it could be hypothesized that transferring hairs onto the plate can increase the chance of obtaining positive cultures. Conversely, hairs are known to also carry spores of contaminant fungi, and the growth of these fungi may negatively affect the interpretation of culture results.[4]
This study was aimed at comparing two inoculation techniques of material collected by the toothbrush method; the first involved purely pressing the toothbrush onto the agar surface, and the second involved pressing the toothbrush onto the agar, as well as transferring hairs and scales removed from the bristles to the plate.

Materials and methods

Study population

The study was conducted on 26 cats housed in a cattery with a history of recurrent dermatophytosis. The cats lived in a rural area where they were allowed to freely roam.

Sampling procedure

Two new, individually-wrapped, human toothbrushes were used for each cat. Each toothbrush was longitudinally combed for 3 min over the hair coat of each cat, starting from the head, followed by the neck, dorsum, trunk, ventrum, limbs and tail. After specimen collection, the toothbrushes were placed in new self-sealing plastic bags and transported to the laboratory of (this information will be provided after the revision of the manuscript).

Evaluation of hairs and scales

Evaluation of the number of collected hairs and scales was carried out in the mycology laboratory by a single investigator before plate inoculation. Examples reported in Figure 1 were used to assist scoring. The quantity of hairs and scales was evaluated as follows:

1. low (barely any visible material with the naked eye)
2. fair
3. abundant
4. very abundant (toothbrush completely covered by hairs entrapped in the bristles)

Fungal cultures

The toothbrushes from each cat were randomly allocated to inoculation procedure A or B using a random choice generator (http://jklp.org/html/choose.html). For procedure A, the toothbrush was pressed onto the surface of the agar (20 repetitions). Even in cases with abundant or very abundant material, it was observed that most hairs and scales remained entrapped in the bristles after pressing the toothbrush on the agar. With procedure B, bristles were stabbed onto the agar surface (20 repetitions). Subsequently, all hairs and scales entrapped in the bristles were removed by flame-sterilized hemostats and pressed gently onto the agar surface.
Cultures were performed on Mycobios Selective Agar (Biolife, Milan, Italy) (formula per litre: soy peptone 10 g; glucose 10 g; cycloheximide 0.4 g; chloramphenicol 0.05 g; agar 15 g). Plates were incubated at 25°C[6] and examined daily for 2 weeks by a mycologist blinded to the inoculation technique. Fungal colonies were identified to species level based on their morphology and microscopic features.[4]

Comparison of the procedures

Results obtained using the two procedures were compared considering the following parameters:

- Number of plates with a positive result (growth of dermatophyte colonies).
- Number of plates with non-dermatophytic contaminant moulds (NDM).
- Number of colony-forming units (CFUs) of dermatophytes and NDM per plate.
- Degree of plate invasion by either dermatophytes or contaminating NDM, calculated through an image processing and analysis program (imageJ, U.S. National Institutes of Health, Bethesda, MD website, imagej.nih.gov/ij/), and expressed as the percentage of plate surface (PPS) invaded by fungal colonies.
- Impact of the degree of plate invasion by contaminating NDM on the ease of visualizing and sampling suspected dermatophyte colonies by microscopic examination. This parameter was rated as follows (see Figure 2 for examples):

    - PPS occupied by NDM < 25%. Visualization and sampling very easy
    - PPS occupied by NDM 25 - 50%. Visualization and sampling easy
    - PPS occupied by NDM 51-80%. Visualization and sampling difficult
    - PPS occupied by NDM >80%. Visualization and sampling very difficult

Statistical analysis

The prevalence of plates with dermatophyte colonies and NDM from the two procedures was compared by the Chi square test, while the Wilcoxon rank-sum test with continuity correction was used to compare the number of CFUs and the PPS. All of the analyses were performed with R Core Team software (2014) (http://www.R-project.org/). A P-value of < 0.05 was considered statistically significant.

Results

A total of 21 cats were culture-positive, with *M. canis* being the only dermatophyte isolated. The quantity of hairs and scales collected on the two toothbrushes from each cat was equivalent in all cases. Specifically, the quantity was rated as low in 4 cases (19%), fair in 2 cases (9.5%), abundant in 10 cases (47.6%) and very abundant in 5 cases (23.8%).
A summary of the culture results is provided in Table 1, while individual results can be found in Table S1 (supplementary material). Procedure A allowed a significantly higher

number of positive plates (20/21; 95.2%) to be obtained compared with procedure B (7/21; 33.3%) ($\chi^2 = 17.53$, $p < 0.01$).There was no significant difference regarding the number of plates with NDM. However, the number of NDM CFUs and the PPS invaded by NDM were significantly higher in plates inoculated using procedure B. Conversely, for *M. canis* the number of CFU and the PPS were significantly higher in plates inoculated using procedure A (Figure S1). Differences were also noted regarding the ease of visualizing and sampling *M. canis* colonies (e.g. 80% of plates were considered easy/very easy in procedure A compared to 43% plates in procedure B, Table 1). However, a statistical comparison for this parameter was not possible due to the low number of positive plates obtained in procedure B.

Discussion

This study shows that the diagnostic value of fungal culture using the toothbrush technique is heavily affected by the way the plate is inoculated. Specifically, transferring hairs and scales from the toothbrush bristles to the agar (procedure B) only allowed isolation of *M. canis* in 33% of cases, while significantly better results could be obtained when the toothbrush was purely pressed onto the agar surface. These results indicate that cultures can be positive even if most material (hairs and scales) remain on the bristles. This is likely due to the fact that very small infected hair fragments and scales, and also free fungal elements (arthroconidia), are transferred to the plate by pressing the toothbrush onto the agar.

Plates inoculated with hairs and scales (procedure B) were frequently invaded by a high quantity of NDM, so that the space in the plate became unavailable for the dermatophyte colonies. For some samples, a nearly complete invasion of the plate by NDM was observed (see Figure 2d and Table S1). The significantly higher invasion of the plate surface by NDM appears to be the main reason for the delusory results obtained by procedure B (only 33% positive plates vs. 95% obtained by procedure A). Inoculating hairs on the medium surface is thus not only unnecessary, but even detrimental. The fact that NDM colonies grew in the plates – in some cases very abundantly – despite the use of a NDM growth inhibitor (cycloheximide) is not, however, surprising. The presence of NDM colonies in cultures from cutaneous samples is a “normal” occurrence in the veterinary laboratory[2–4,8], since the animal hair coat harbours a variegated fungal flora[8], and cycloheximide is not equally effective against all NDM species.[9]

Another advantage of procedure A is that the abundance of *M. canis* colonies, coupled with the scarce NDM contamination, made it easy or very easy in most positive plates (80%) to visualize and sample the colonies for microscopic confirmation. It should also be noted that the number of *M. canis* colonies is a parameter that helps discriminating between animals exposed to fomite contamination and cats with an active infection. It is also useful to monitor the course of infection during treatment.[3] Regarding procedure B, in addition to the already mentioned overall poor performance, in more than half of the positive plates (57%) the individuation and sampling of suspected colonies resulted difficult or very difficult.

In conclusion, this study provides evidence that the correct technique to inoculate fungal cultures when using the toothbrush technique consists of stabbing bristles onto the agar without plating hairs and scales plucked from the bristles.

Supplementary material

Table S1. Individual results of cultures using procedure A and procedure B

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Table legend

Table 1. Results of cultures obtained using two different procedures of inoculation

Figure legends

Figure 1. Evaluation of the quantity of hairs and scales collected after brushing. Examples of (1) Low quantity. (2) Fair quantity. (3) Abundant quantity. (4) Very abundant quantity.

Figure 2. Examples of culture plates obtained in the study. Visualization and sampling of suspected *M. canis* colonies assessed as: a) very easy; b) easy; c) difficult; d) very difficult. Colonies marked with * = *M. canis*. Colonies marked with ° = non dermatophytic moulds (NDM)